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14. ABSTRACT

Pain is a common and distressing symptom that impacts the quality of life of many patients with neurofibromatosis. The pain is often due to the formation of a neuroma. To understand better how neuromas cause pain and what treatments may be provided, we have developed an animal model of a painful neuroma. The tibial neuroma transposition (TNT) model has been confirmed as a model of neuropathic pain. The TNT model has been established as reliable and valid (Specific Aim 1). In the TNT model, the neuroma test-site mechanosensitivity is dependent on neural input from the tibial neuroma. In the TNT model, hindpaw mechanical hyperalgesia is independent of input from the tibial neuroma. We have altered the formation of a neuroma by applying a toxin that is retrogradely transported (suicide transport) leading to neuronal death and axonal death (Specific Aim 2). This technique is now being refined using target-specific toxins and examining subsequent pain behaviour (Specific aim 3).

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INTRODUCTION

A percentage of NF1 patients may experience an increase in pain after surgical removal of a neurofibroma. This pain is due to the formation of a painful neuroma, a jumbled mass of nerve fibers and connective tissues, at the cut end of the nerve. Palpating the tissue overlying a neuroma evokes paresthesias/dysasethesias in the distribution of the injured nerve. Surgical resection of the neuroma may provide relief, but the pain often recurs following the inevitable evolution of a new neuroma at the nerve end. Previous animal models of neuropathic pain have focused on the mechanical hyperalgesia and allodynia that develops at a location distant from the site of injury and not on the pain from direct stimulation of the neuroma. We describe a new animal model of neuroma pain, the tibial neuroma transposition (TNT) model, in which the neuroma is located in a position that is accessible to mechanical testing and outside of the innervation territory of the injured nerve. This allows testing of pain in response to mechanical stimulation of the neuroma (which we call neuroma tenderness) independent of pain due to mechanical hyperalgesia. Mechanical stimulation of the neuroma produced a profound withdrawal behavior that could be distinguished from the hyperalgesia that developed on the hindpaw. The ultimate objective of this research is to prevent reformation of a painful Neuroma by using suicide transport of neuronal toxins.

BODY

We will present a summary of our efforts that represent 1, research based directly on the specific aims of the grant and 2, outgrowth research to improve methodology in this work and increase our understanding of the patho-physiology underlying neuropathic pain.

1) Specific Aim Directed Research

In year one, we firmly established the TNT model with the addition of sufficient animal numbers to our preliminary work to produce a reliable, statistical and publishable result. We then completed our first specific aim by demonstrating that blocking neural input from the neuroma to the CNS reversed the pain behavior produced by the TNT model. In year 2 we experimented with a variety of neural toxins to prevent neuroma formation through retrograde transport and cell death. In year 3 we continued experimenting with a variety of neural toxins and delivery methods to achieve suicide transport and reverse pain behavior.

A) Specific Aim Direct Research

1) **Specific aim #1: Does blocking neural input from the neuroma to the CNS reverse the pain behaviors produced by the TNT model?** As indicated in the previous progress reports, this specific aim has been completed.

2) **Specific aim #2: Develop a technique to selectively prevent neuroma formation with OX7-saporin.** As indicated in the year two progress report, we did not obtained a reproducible decrease in behavioral signs of pain when OX7-saporin was injected into the nerve. During the past year, we have explored two different strategies to overcome this difficulty. The first strategy was to use different neural toxins. The second strategy was to employ different techniques for administering the neural toxins. A brief summary of the results so far with these experiments is provided below.

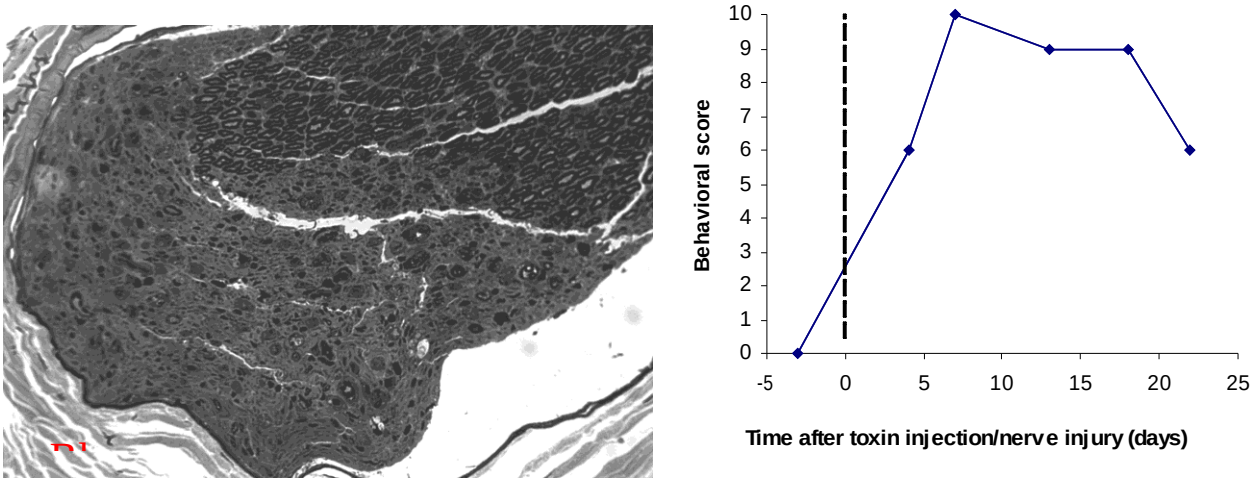
a. <u>Use of different neural toxins.</u> After discussion with various experts in the field of neural toxins and retrograde transport, we identified two other neural toxins that may be effective: Wheat Germ Agglutinin (WGA) coupled to saporin and cholera toxin B (CTB) coupled to saporin. WGA binds to unmyelinated fibers and should lead to selective loss of unmyelinated fibers. CTB binds to large myelinated fibers and should lead to a selective loss of myelinated fibers.

WGA-saporin was injected into the tibial nerve (in doses ranging from 5 to 200 ng in 2 ul), the nerve was ligated distal to the injection and rotated to the lateral position (using our standard approach for producing the TNT model). Behavioral testing 1 to 3 weeks showed variable results. Most animals, showed little

evidence for an analgesic effect of the injection. Even at the higher doses, some animals showed modest anaglesia and others none at all. Despite the lack of reproducible behavioral effects, the histological samples from the proximal nerve showed evidence for degeneration following the neural toxin. Similar results were obtained when CTB-saporin (in doses ranging from 0.03 to3.0 ug in 2 ul) was injected into the tibial nerve.

The pain behavior from neuroma formation did not reverse when either CTB or WGA toxin was employed. It was felt that preservation of either the mylinated or unmyelintaed could be signaling the pain and thus we decided to move ahead with new series of experiments. We tried a mixture of CTB and WGA at various doses in an attempt to prevent regeneration of both classes of fibers. This still did not consistently reverse the pain behavior.

A "breakthrough" in our thinking on this came when we investigated histological samples taken close to the neuroma site (i.e., 3 mm proximal to the ligature). The figure below shows the results in one animal following injection of 3 ug of CTB-saporin into the tibial nerve. As shown

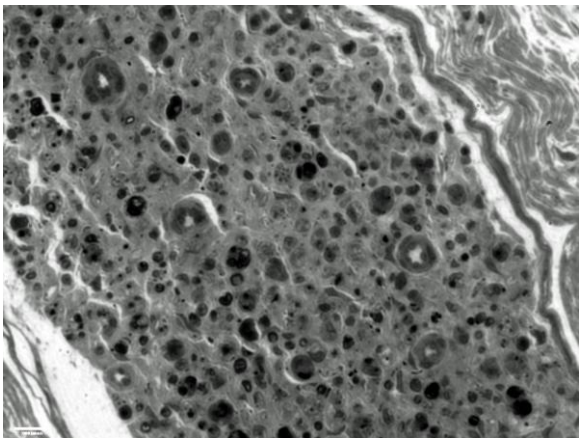


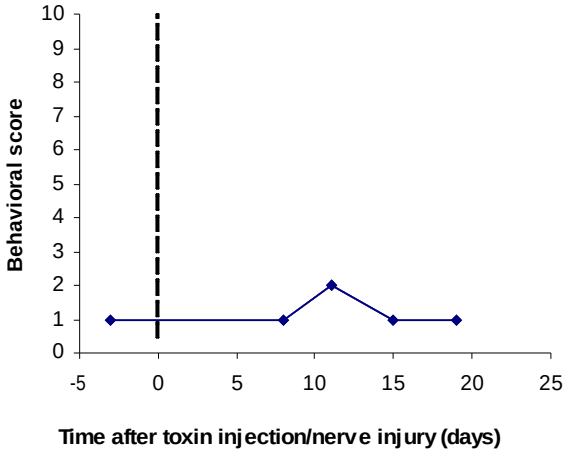
in the left panel, pronounced degeneration is seen in part of the nerve, but the other part of the nerve is relatively spared. This suggests that the micro-injection of the neural toxin was restricted to one fascile in the nerve and the toxin did not cross over to adjacent fasicles. In the right panel, the behavioral response following mechanical stimulation at the neuroma site is plotted as a function of time after nerve injury (and toxin injection). This animal reach the maximum behavioral score (i.e., 10) and stayed at a high behavioral score throughout the three week testing period. Thus, no obvious signs of analgesia were apparent. Our interpretation of these results is that the spared fascile innervated the neuroma and provided sufficient neural signaling to produce the behavioral response. Base on this observation, we set out to develop alternate techniques for administering the neural toxins that would lead to a complete denervation of the tibial nerve and reversal of pain behavior (see next section).

b. <u>Development of different techniques for administering the neurotoxins.</u>

The first idea was to crush the nerve prior to injecting the neural toxin. It appeared that the neural toxin was respecting the perineurial barrier. We reasoned that the crush would disrupt the perineurium and provide access of the neural toxin to all of the fasicles. This technique did not result in improved behavioral responses.

The next idea was to micro-inject each of the fasicles in the tibial nerve. An example of the outcome of this experiment in one animal is shown below.





In this case, there was complete degeneration of the tibial nerve (left panel) and only a weak behavioral response (right panel). However, this procedure proved to be technically very challenging since it was difficult to insert the needle into some of the smaller fasicles. We still had animals that had incomplete degeneration and showed no behavioral signs of analgesia.

Our most recent idea is to place the cut nerve into a pool (or "well") of neurotoxin solution. This would expose all fasicles to the neural toxin. To achieve this, the tibial nerve is cut and ligated. The suture is used to pull the nerve thru a PE50 tubing. The nerve and tubing is then cut proximal to the ligature and the nerve is pulled back into the tubing so that a 1.5 mm empty tubing space is formed that serves as a drug loading pool just distal to the nerve stump. The distal end of the tubing closed. A glass micropipet is used to load this space with the toxin. The nerve is exposed to the toxin for 1 - 2 hours. Then, the tubing is removed, and the nerve is ligated and rotated to the lateral position (as per our normal TNT procedure). Our initial results with this technique have been promising. A series of experiments have been started using a dose response escalation. We expect the complete result of these experiments to be available in the next few months. This work is being completed during the no-cost extension period.

3) **Specific aim #3: Does OX7-saporin prevent or reverse the pain behaviors produced by the TNT model?** This specific aim will be quickly completed if we can resolve the issues that we uncovered in attempting to perform specific aim 2. We measure both neuroma formation and behavior when doing each of our experiments. We have found that if the neural toxin does not result in preventing any neuroma formation, the behavior persists

2) Outgrowth Research

This grant has lead to the development of the TNT animal model of neuroma pain. There have been numerous investigators wanting further information on the model. A number have already replicated the model in their laboratories and begun investigations.
We have begun collaboration with Michel Kliot MD, Professor Dept. of Neurosurgery, University of Washington. He is the chief of neurosurgery at Puget Sound VA Health Care Center. He and his co-investigators have submitted a grant to the Vetrans Adminstraion. The following is a relevant portion.

Our long-term goal is to change the paradigm of how patients presenting with pain due to focal damage to peripheral tissues are diagnosed. We have developed a new focused ultrasound (FUS) based technology that we call transcutaneous acoustic palpation (TAP) that promises to be far more specific than physical examination and diagnostic imaging in identifying pain generators that are deep within the body. We have already demonstrated in two animal models generating superficial sources of pain that FUS can reliably distinguish the tender from the non-tender extremity. We have also demonstrated that we can apply FUS under ultrasound-image guidance. **As a next logical step, we propose to demonstrate that FUS can identify a deeper source of focal pain using the subcutaneous *tibial neuroma transposition model* developed by Belzberg and colleagues (Dorsi et al 2007).**

We are very excited by this collaboration. It is possible that this technique of localizing a deep pain generator can be applied to differentiating which tumor in a patient with NF1 is the one causing pain.

KEY RESEARCH ACCOMPLISHMENTS

- The formation of a neuroma subsequent to axotomy can be altered by using retrograde transport of a neural toxin in the proximal stump.

- Neuroma test-site mechanosensitivity can be altered by retrograde transport of a neural toxin.

- The pain behavior associated with neuroma formation may not be dependent on ongoing activity in large fiber neurons (A-beta fibers).

- The pain behavior associated with neuroma formation may not be dependent on ongoing activity in small fiber neurons (C-fibers, A-delta fibers).

REPORTABLE OUTCOMES

Dr. Belzberg was the invited guest speaker at the America Society of Peripheral Nerve annual meeting held in Hawaii, January 2009. He provided the *presidential guest lecture* entitled

Neuropathic Pain: from bench to bedside and back again

The work of this grant was heavily featured in the talk and the DOD grant / support acknowledged.

CONCLUSION

The pain behavior displayed by the animal results from mechanical stimulation of the neuroma, a phenomenon commonly seen in patients with painful neuroma. The tibial neuroma transposition (TNT) model provides the scientific community an animal model of neuroma pain.

The application of Ricin to the nerve will result in retrograde transport of the neural toxin and axonal degeneration. There is a dose dependent loss of axons and prevention of neuroma formation.
The application of Wheat Germ Agglutinin – SAP to a nerve will result in retrograde transport of the neural toxin and loss of small fiber axons. The loss of these "pain fibers" did not result in a loss of pain behavior. The application of cholera toxin B (CTB) coupled to saporin to a nerve will result in retrograde transport of the neural toxin and loss of large fiber axons. The loss of these fibers did not result in loss of pain behavior. It appears that incomplete lesions of the nerve were performed with the application of the neural toxins. We are proceeding with a novel technique of drug delivery using a pool.

Utilizing a variety of drug delivery techniques we will determine if we can both prevent painful neuromas from forming and reverse pain behavior by treating an existing neuroma.

Appendices

Supporting Data